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Anti-inflammatory and antithrombotic effects of nicotine exposure in oral contraceptive-induced insulin resistance is glucocorticoid-independent

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Running Title: Anti-inflammatory and antithrombotic effects of nicotine during COC use

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ABSTRACT

Background: Reports showed that estrogen-progestin oral contraceptive (COC) or tobacco smoking causes increased risk of cardiovascular diseases (CVD) in premenopausal women. Studies also suggest that nicotine; a major tobacco alkaloid may worsen or improve atherothrombotic CVD. Altered hemorheology, prothrombotic and pro-inflammatory biomarkers, have been implicated in the development of atherothrombotic CVD events. However, the effect of non-smoking nicotine exposure on these biomarkers during COC treatment is not yet established. We therefore sought to determine the effects of nicotine exposure during COC treatment on these biomarkers, and also tested the hypothesis that the nicotine effects would be glucocorticoid-dependent.

Methods: Female Sprague-Dawley rats aged 10 weeks were given (*po*) vehicle, low-dose nicotine (0.1 mg/kg) or high-dose nicotine (1.0 mg/kg) with or without COC steroids (5.0 µg/kg ethinylestradiol and 25.0 µg/kg levonorgestrel) daily for 6 weeks.

Results: COC treatment or nicotine exposure led to increased insulin resistance (IR), hemorheological (blood viscosity, hematocrit and plasma viscosity), prothrombotic (plasminogen activator inhibitor-1), pro-inflammatory (uric acid, C-reactive protein, neutrophil/lymphocyte and platelet/lymphocyte ratios) biomarkers and corticosterone. However, these effects except that on corticosterone were abrogated by nicotine exposure during COC treatment.

Conclusions: Our study indicates that nicotine- or COC-induced IR may be mediated via inflammatory/thrombotic pathway. The results imply that nicotine exposure could impact negatively on atherothrombotic biomarkers in COC non-users, whereas the impact in COC users could be positive. The results also suggest that the anti-inflammatory, antithrombotic and blood viscosity-lowering effects of nicotine exposure during COC use is circulating glucocorticoid-independent.

Keywords: Atherothrombotic CVD, estrogen-progestin oral contraceptive, proinflammatory biomarker, corticosterone

Introduction

Cardiovascular, renal, and metabolic disorders are inextricably linked and are the leading causes of mortality and morbidity globally. Among metabolic dysfunctions, type 2 diabetes/insulin resistance (IR) ranks first with 23% surge in prevalence over the last decade, it now affects more than 29.1 million people [1]. Despite increased efforts to prevent, treat, and control IR and its sequelae, the prevalence has not declined. IR is a condition in which there is reduced sensitivity of organs to insulin-initiated physiologic processes that result in metabolic defects. IR occurs in 20–25% of the human population [2] and it is a hallmark of both the prediabetic state and overt type 2 diabetes. It is also a critical metabolic link among hypertension, dyslipidemia, type 2 diabetes, obesity and atherothrombotic cardiovascular disease (CVD) [3]. Although the incidence of CVD is considerably lower in premenopausal women than in age-matched men or postmenopausal women [4], however, there are reports that young women may have increased risk factors for atherothrombotic CVD during hormone therapy [5–7].

Atherosclerosis is a long-term, progressive disease process that frequently leads to an acute atherothrombotic event through plaque rupture and formation of a platelet-rich thrombus. The principal clinical manifestations of atherothrombosis are sudden cardiac death, myocardial infarction, ischemic stroke, and peripheral arterial ischemia. Studies have revealed that atherothrombotic events are the end result of a complex inflammatory response to multifaceted vascular pathology [8]. Inflammation plays a central role in the pathogenesis of atherothrombotic events [9]. Among the inflammatory cytokines, C- reactive protein (CRP) has been identified as a non-specific but sensitive biomarker of the inflammation-mediated atherothrombotic events [10].

Altered blood rheology has been linked to the development of atherothrombotic complications and increased systemic inflammation seems to be an important clinical correlate [11, 12]. Studies have shown that altered levels of blood rheological markers, which affect blood flow in both macro-and micro-circulations, are associated with incident atherothrombotic CVD. These markers include blood viscosity and its major determinants: hematocrit and plasma viscosity [11]. White blood cells, particularly increased polymorphonuclear and decreased lymphocytes are considered as markers of inflammatory-mediated atherothrombotic CVD [13–15]. Platelets also play a major role in this inflammation-mediated atherothrombotic process [15] and platelet to lymphocyte ratio (PLR) has been reported as another reliable marker of inflammation-mediated atherothrombotic events [16, 17].

Over 10% of cardiovascular deaths are thought to be caused by cigarette smoking [18] and more than two-thirds of sudden cardiac deaths resulting from acute atherothrombosis occur in smokers [19]. Notably, the exposure to secondhand cigarette smoke has been associated with a larger risk of cardiovascular mortality than would be expected on the basis of linear extrapolation of the relative risk from active smoking [20]. Cigarette smoking has also been associated with altered hemorheology [11], pro-inflammatory and prothrombotic responses [21–23]. Nicotine, a predominant chemical among over 4000 compounds in cigarette smoke, is widely used as nicotine replacement therapy (NRT) either as gum or patch preparations for smoking cessation [24]. Studies have shown that nicotine is beneficial in diseases with inflammation-related mechanism such as experimental type 1 diabetes [25], sepsis [26], ulcerative colitis [27], and renal ischemia-reperfusion injury [28]. Results from clinical or animal studies are conflicting regarding effects of nicotine on cardiometabolic disorders and atherothrombosis [11, 21, 28–33].

Glucocorticoids, like cortisol in humans and corticosterone in rodents, are stress and catabolic hormones which have strong anti-inflammatory properties, are highly effective in the treatment of allergies and inflammatory conditions [34]. They are primarily responsible for mobilizing glucose to the circulation from the liver and also by inhibiting the uptake and utilization of glucose. However, individuals with elevated circulating glucocorticoids present a variety of systemic manifestations that are associated with increased atherothrombotic CVD risk, such as increased adiposity, hypertension, IR/glucose intolerance, diabetes mellitus, and dyslipidemia [35, 36]. Currently, studies have suggested that glucocorticoids markedly increased circulating plasminogen activator inhibitor-1 (PAI-1) [37–39]. PAI-1 is a serine protease inhibitor that primarily inhibits fibrinolysis by inactivating urokinase-type and tissue-type plasminogen activator [40]. It is a prothrombotic biomarker and an independent risk factor for atherothrombotic CVD [41–43]. Elevated circulating level of PAI-1 is found in inflammation-related atherothrombotic events [42, 43]. Nicotine has also been linked to increased production of PAI-1[29]. Moreover, we recently demonstrated in female rats with IR induced by COC [44], elevated levels of circulating PAI-1, cardiac PAI-1 mRNA and protein.

Since COC is apparently used for long duration by young healthy women and nicotine exposure is increasing in young women. It is interesting to note that studies evaluating the effects of non-smoking nicotine exposure during COC use is lacking, particularly on pro-inflammatory, hemorheological and prothrombotic biomarkers. This study therefore aimed at investigating the effects of nicotine exposure on hemorheological, prothrombotic and pro-inflammatory biomarkers during COC treatment. We sought to test the hypothesis that nicotine or COC induces IR through inflammatory/thrombotic pathway. We also aimed at

testing the hypothesis that the nicotine effects with or without COC treatment would be glucocorticoid-dependent.

Materials and Methods

Animals

The investigation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Ethical Review Board of University of Ilorin, and every effort was made to minimize both the number of animals used and their suffering. Ten weeks old female Wistar rats were obtained from the animal house of the College of Health Sciences, University of Ilorin (Ilorin, Nigeria). Rats had unrestricted access to standard rat chow and tap water. After one week of acclimatization, the animals were randomly assigned to 6 groups (n=6/group). Rats were maintained under standard environmental conditions of temperature, relative humidity, and 12-hour dark/light cycle.

Treatment

Control group received distilled water (vehicle; *po*) daily. COC-treated group received (*po*) a combination of 5.0 µg/kg ethinylestradiol and 25.0 µg/kg levonorgestrel (Wyeth–Ayerst, Inc., Montreal, Canada) daily. Low NIC group received (*po*) 0.1 mg/kg nicotine (Alchem Inc., Minnesota, USA) daily, High NIC group received (*po*) 1.0 mg/kg nicotine daily whereas, COC+Low NIC group received (*po*) 0.1 mg/kg nicotine plus COC steroids (5.0 µg/kg ethinylestradiol and 25.0 µg/kg levonorgestrel) daily and COC+High NIC group received (*po*) 1.0 mg/kg nicotine plus COC steroids (5.0 µg/kg ethinylestradiol and 25.0 µg/kg levonorgestrel) daily. The treatment lasted for 8 weeks.

Sample preparation

At the end of treatment, the rats were anesthetized with pentobarbital sodium after 12 h overnight fast. Blood was collected by cardiac puncture into EDTA-coated and heparinized bottles, accordingly. Blood collected in heparinized bottle was centrifuged at 3000 rpm for 5 min. Plasma was stored frozen until needed for biochemical assay.

Insulin resistance (IR)

Plasma Insulin was determined using ELISA kit from Ray Biotech, Inc. (Georgia, USA), whereas fasting glucose was analyzed by glucometer (ONETOUCH®-LifeScan, Inc., Milpitas, CA, USA). IR was estimated using the homeostasis model assessment for IR (HOMA-IR) HOMA-IR was expressed as $[\text{fasting glucose (mmol/l)} * \text{fasting insulin (}\mu\text{U/l)}]/22.5$. Insulin sensitivity was determined using quantitative insulin sensitivity check index (QUICKI) expressed as $1/[\log(\text{fasting insulin}) + \log(\text{fasting glucose})]$.

Hematological and hemorheological parameters

Blood collected in the EDTA-coated bottles were used for hematological analysis through a multi-parameter, automated hematological analyzer, URIT-3010 (Xianyang, China). Plasma viscosity was determined by adapted capillary viscometry. The temperature was maintained at 37°C in a thermostatic incubator (BISCA, model Cella, Thermostica Ad-aqua, BE-89, Italy). Relative plasma viscosity was estimated as previously described in earlier study [45] whereas blood viscosity was estimated by modified Vand formula [45,46].

Biochemical assay

Plasma high sensitive C- reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1) were determined using ELISA kit from Cloud-Clone Corp. (Texas, USA). Plasma level of corticosterone, an anti-inflammatory biomarker, was determined using ELISA kit obtained

from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Plasma uric acid was measured by standardized enzymatic colorimetric method using assay kit obtained from Fortress Diagnostics Ltd. (Antrim, UK). Platelet/lymphocyte (PLT/LYM) and polymorphonuclear/lymphocyte (PMN/LYM) ratios were also estimated as marker of inflammation.

Statistical analysis

All data were expressed as means $\pm$ SEM. Statistical group analysis was performed with SPSS statistical software. One-way analysis of variance (ANOVA) was used to compare the mean values of variables among the groups. Bonferroni's test was used to identify the significance of pairwise comparison of mean values among the groups. Statistically significant differences were accepted at $p < 0.05$.

Results

Effects of oral nicotine with or without COC treatment on body weight gain, food intake, regional adiposity, fasting glucose, insulin and insulin sensitivity

COC treatment or high nicotine led to significant decrease in body weight gain whereas body weight gain was not affected by nicotine exposure during COC treatment (Fig. 1A). High nicotine led to significant decreased food intake (Table 1). Nicotine at both doses with or without COC treatment led to significant decreases in regional adiposity in a dose-dependent manner whereas COC treatment led to a significant increase in regional adiposity (Fig. 1B). Fasting glucose was comparable in all the experimental groups. High oral nicotine and COC treatment led to significant increase in fasting insulin compared to control. Concomitant oral nicotine administration at both doses with COC treatment significantly abrogated increased fasting insulin (Table 1). COC treatment or oral nicotine at both doses led to significant

increased HOMA-IR whereas oral nicotine with COC treatment normalized HOMA-IR (Fig. 1C and D).

Effects of oral nicotine with or without COC treatment on hematological and hemorheological parameters

COC treatment led to significant increases in hematocrit, hemoglobin concentration and platelet count compared to the controls. Low nicotine exposure led to significant increases in total white blood cell and lymphocyte counts, whereas nicotine exposure with COC treatment resulted in significant abrogation in hematocrit, hemoglobin level, red blood cell and platelet count when compared to COC-treated rats (Table 2 and Fig. 2B). COC treatment or nicotine exposure at both doses led to significantly increased blood and relative plasma viscosities compared to the controls. There was no significant difference in blood and relative plasma viscosities when nicotine was administered at both doses during COC treatment when compared with the controls (Fig. 2A and C).

Effects of oral nicotine with or without COC treatment on pro-inflammatory and prothrombotic biomarkers

COC treatment or nicotine exposure led to significant increases in plasma uric acid, CRP and PAI-1 (Figs. 3A, B and 4), whereas COC treatment led to significant increases in PMN/LYM and PLT/LYM ratios. However, nicotine exposure at both doses with COC treatment did not result in any significant alterations in the pro-inflammatory and prothrombotic biomarkers when compared with the control values (Figs. 3 and 4).

Effects of oral nicotine with or without COC treatment on circulating glucocorticoid

COC treatment and oral nicotine at both doses with or without COC treatment led to significant increase in plasma corticosterone level compared to control (Fig. 5).

Discussion

In the current study we sought to investigate the estrogen-progestin COC- and circulating glucocorticoid-dependence of chronic oral nicotine on atherothrombotic CVD risk factors in a female rat model of cardiometabolic disorder [44]. Our data showed that oral nicotine produces divergent effects on blood rheological, prothrombotic and pro-inflammatory biomarkers with or without COC treatment. Our data demonstrate that oral nicotine or COC treatment led to increased blood rheological, prothrombotic and pro-inflammatory biomarkers, whereas these alterations were abrogated by oral nicotine with estrogen-progestin COC treatment. The results also indicate that the anti-inflammatory and antithrombotic effects of oral nicotine during COC treatment is accompanied by improved blood rheology, IR and enhanced circulating corticosterone, an anti-inflammatory glucocorticoid. In these COC-treated female rats as in a male mouse model of renal ischemia-reperfusion injury [26], nicotine appears to act as a potent anti-inflammatory agent, and by extension as an anti-thrombotic agent by reducing circulating PAI-1, reflecting a cardioprotective effect of nicotine in female rats treated with estrogen-progestin COC. Since nicotine exposure without COC treatment resulted in dose-dependent abnormal hemorheology, pro-inflammatory and prothrombotic effects, while nicotine exposure in COC-treated rats led to amelioration of these effects provides evidence that the cardioprotective effect of nicotine is ovarian hormones-dependent as recently suggested in ovariectomized rats [47].

The finding that COC treatment in these animals resulted in decreased insulin sensitivity that is accompanied by reduced body weight gain and increased regional adiposity suggest that COC-induced IR is associated with accumulated regional fat regardless of body weight gain. However, IR caused by oral nicotine in this present study is associated with reduced body

weight gain and regional adiposity in dose-dependent manners, implying that nicotine-induced IR is not associated with body weight gain or increased regional fat accumulation.

These findings further imply that IR/cardiometabolic disorder induced by oral nicotine or COC treatment is associated with similar effect on body weight gain but diverse effect on regional fat accumulation. The observation that COC-induced IR is consistent with previous studies in experimental animals in our laboratory [44] as well as in humans [48]. Reduced insulin sensitivity, body weight gain, and regional fat accumulation by oral nicotine observed in female rats in the present study are in consonance with earlier observation by other workers [30, 33].

Cardiometabolic syndrome is a cluster of interrelated metabolic abnormalities that include atherogenic dyslipidemia, elevated blood pressure, glucose dysregulation, increased adiposity, inflammation and thrombosis with IR [49]. Several meta-analyses have shown that individuals with cardiometabolic disorder have at least 2-fold increased risk of developing atherothrombotic CVD events and type 2 diabetes [50]. IR has been suggested to be a major metabolic link [3]. Hence, intervention that could promote or prevent the development of IR and its attendant atherothrombotic CVD risk factors would be of great public health importance, particularly in COC users.

Atherothrombotic CVD is a result of inflammatory vasculopathy including sudden cardiac death, myocardial infarction (MI), ischemic stroke, and peripheral arterial ischemia [8]. Since report exists that estrogen-progestin hormonal use in young and old women has been linked to increased incident atherothrombotic CVD [5-7] and that over 10% of cardiovascular deaths are thought to be caused by nicotine exposure particularly through cigarette smoke and more than two-thirds of sudden cardiac deaths resulting from acute atherothrombosis occur in

smokers [18,19]. Hence, exposure to nicotine during COC treatment should be of a great importance. The findings in the present study that COC treatment led to abnormal blood rheology is in consonant with earlier data from our laboratory animals [45] and those from clinical studies [11]. Our findings in the current study that COC treatment caused increased pro-inflammatory biomarkers that are accompanied by altered blood rheology and prothrombotic marker is corroborated by some studies among women using COC [11]. Similarly, oral nicotine caused increased pro-inflammatory markers accompanied by altered blood rheology and prothrombotic marker is consistent with previous studies [21,29]. The fact that evidence exist that increased pro-inflammatory markers [11,12], altered blood rheology, and prothrombotic marker (PAI-1) play considerable pathogenic role in the development of IR may suggest that IR induced by COC or nicotine exposure may be due to inflammation and thrombosis. The study also provide further evidence that non-smoking oral nicotine and COC use are independent risk factors for the development of atherothrombotic CVD and type 2 diabetes.

It is interesting to also note that IR and increased atherothrombotic CVD risk factors induced by oral nicotine or COC treatment are associated with elevated circulating glucocorticoid levels. Since, elevated circulating glucocorticoids have been linked to the development of inflammatory mediated atherothrombotic CVD [35,36]. Recent studies have suggested that glucocorticoids markedly increased circulating PAI-1 [37–39]. It may be plausible to suggest that elevated glucocorticoids play considerable role in the development of IR and associated atherothrombotic CVD risk markers induced by oral nicotine or COC treatment in this experimental female rat model. Admittedly, oral nicotine or COC treatment caused reduced insulin sensitivity at comparative magnitude in this animal model.

However, our data from these animals indicate that COC treatment induced comparative increased blood rheology, stronger increased pro-inflammatory markers and lesser prothrombotic marker when compared with the effects of oral nicotine on these markers, suggesting differential contribution of these markers to reduced insulin sensitivity induced by oral nicotine or COC treatment. The observation that COC-induced reduced insulin sensitivity might be mediated by a stronger contribution of pro-inflammatory markers, may be explained by the fact that COC treatment led to increased regional adiposity, whereby nicotine exposure induced reduced insulin sensitivity received stronger contribution from the prothrombotic markers which might be explained by stronger circulating levels of glucocorticoid [37–39].

Another interesting finding in the current study is that oral nicotine at both low and high doses abrogated the development of IR and accompanied increased blood viscosity, PAI-1, and pro-inflammatory markers. These findings imply that oral nicotine or COC administered singly and when administered concomitantly, would impact divergent effects on insulin sensitivity as well as atherothrombotic CVD risk markers. However, reports exists that COC and smoking-derived nicotine can synergistically increase the risk and incidence of myocardial infarction, peripheral thrombus formation and ischemic stroke [51]. To the best of our knowledge, this is the first study that report the effect of non-smoking derived nicotine exposure on inflammatory, thrombotic and blood rheological markers during COC treatment. It is surprising to find that oral nicotine abrogated increased pro-inflammatory, blood viscosity and prothrombotic markers in COC-treated female rats.

However, the finding regarding the abrogative effects of nicotine on pro-inflammatory markers is corroborated by studies in experimental rodents that indicate that non-smoking

nicotine has anti-inflammatory effect [52,53]. The result from this current study that implicated beneficial effect of nicotine on blood rheology during COC treatment is also in consonance with a previous study that suggested that nicotine replacement improves blood rheological properties [21].

More so, the findings that elevated circulating glucocorticoid levels observed in COC-treated rats was enhanced by oral nicotine whereas increased regional adiposity induced by COC use was abrogated by oral nicotine, may suggest that abrogative effect of nicotine on atherothrombotic CVD risk factors during COC use is independent of glucocorticoid.

Hence, monitoring of circulating glucocorticoid as an anti-inflammatory or atherothrombotic marker in COC users exposed to nicotine may not be effective.

Conclusions

We have shown that COC use or nicotine exposure could lead to reduced insulin sensitivity that is accompanied with increased atherothrombotic CVD risk factors. However, the current study demonstrates that oral nicotine administration during COC use would impact positively on atherothrombotic CVD risk factors regardless of the elevated circulating levels of glucocorticoid. The anti-inflammatory and anti-thrombotic potentials of non-smoking nicotine exposure may be a pharmacological target for atherothrombotic CVD events in COC users.

Conflict of Interest

The authors declare no conflict of interest.

Funding Information

The authors have to funding information to declare.

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Figure Legend

Fig. 1. Effect of nicotine (NIC) exposure during COC treatment body weight gain (A) regional body fat (B), HOMA-IR (C), QUICKI (D). COC treatment and high oral nicotine led to significant decrease in body weight gain. Oral nicotine at both doses with or without COC treatment led to significant decrease in regional adiposity in a dose dependent manner whereas COC treatment led to significant increase in regional adiposity. COC treatment and oral nicotine led to significant increase in HOMA-IR, whereas high oral nicotine with COC treatment led to significant decrease in HOMA-IR. COC treatment and high oral nicotine led to significant decrease in insulin sensitivity. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SEM of 6 rats per group (* p <0.05, ** p <0.01 vs. control, # p <0.05, ## p <0.01 vs. COC).

Fig. 2 Effect of nicotine (NIC) exposure during COC treatment blood viscosity on (A) Hematocrit (Hct) (B), Relative plasma viscosity (RPV) (C). COC, Low NIC, High NIC treatment led to increased relative plasma viscosity and blood viscosity. Increased hematocrit levels were observed during COC treatment. COC+ Low NIC and COC+ High NIC attenuate blood viscosity and hematocrit level. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SEM of 6 rats per group (* p <0.05 vs. control, # p <0.05 vs. COC).

Fig. 3 Effect of nicotine (NIC) exposure during COC treatment on circulating pro-inflammatory markers; (A) uric acid (UA) (B) CRP level (C) Polymorphonuclear / Lymphocyte (PMN/LYM) and (D) Platelet/ Lymphocyte (PLT/LYM). COC treatment led to increased UA, CRP, PMN/LYMP and PLT/LYMP levels but Low NIC and High NIC result in increased UA and CRP levels. COC+Low NIC and COC+High NIC attenuate pro-inflammatory markers. Data were analyzed by one-way ANOVA followed by Bonferroni

post hoc test. Values are expressed as mean $\pm$ SEM of 6 rats per group (* p <0.05, * p <0.01 vs. control, # p <0.05, ## p <0.01 vs. COC).

Fig. 4 Effect of nicotine (NIC) exposure during OC treatment on plasma plasminogen activator inhibitor-1 (PAI-1) level. Low NIC, High NIC and COC treatment led to increased PAI-1 levels while COC+ Low NIC and OC+ High NIC treatment attenuated PAI-1 levels. Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc test*. Data are expressed as mean $\pm$ SEM of 6 rats per group (* p <0.05, * p <0.01 vs. control, # p <0.05, ## p <0.01 vs. COC).

Fig. 5 Effect of nicotine exposure during COC treatment on circulating corticosterone. COC treatment and oral nicotine at both doses with or without COC treatment led to significant increase in circulating corticosterone level compared to control. Oral nicotine at both doses led to a dose dependent increase in circulating corticosterone level. High oral nicotine administration during COC treatment led to significant increase in circulating corticosterone level compared to COC treated rats. Data were analyzed by one way ANOVA followed by Bonferroni *post hoc test*. Values are expressed as mean $\pm$ SEM of 6 rats per group (* p <0.05, ** p <0.01 vs. control; # p <0.05 vs. COC).

Fig. S1 Effect of nicotine exposure during COC treatment on cotinine
High dose nicotine exposure with or without COC treatment led to significant increase in cotinine level compared to control. Cotinine was not detectable (N.D) in control and COC treated rats which were not exposed to nicotine. Data were analyzed by one way ANOVA followed by Bonferroni *post hoc test*. Values are expressed as mean $\pm$ SEM of 6 rats per group (** p <0.01 vs. control).

Fig. S2 Effect of nicotine exposure during COC treatment on circulating estradiol

COC treatment and high dose nicotine exposure with or without COC treatment led to significant decrease in circulating estradiol compared to control. Data were analyzed by one way ANOVA followed by Bonferroni *post hoc* test. Values are expressed as mean $\pm$ SEM of 6 rats per group (* p <0.05 vs. control).

Table 1

Effect of nicotine exposure during COC treatment on body weight, food intake, fasting blood glucose, fasting plasma insulin and insulin resistance.

	Control	Low Nicotine	High Nicotine	COC	COC+ Low Nicotine	COC+ High Nicotine
Body weight (g)	152.9 ± 6.5	132.9 ± 6.9	130.0 ± 5.9*	128.3 ± 5.4*	138.2 ± 5.8	134.6 ± 7.8
Food intake (g/kg/day)	74.0 ± 3.0	70.7 ± 6.6	65.2 ± 2.2*	73.5 ± 6.4	83.5 ± 3.2	70.6 ± 6.2
Fasting blood glucose (mmol/l)	4.5 ± 0.1	4.8 ± 0.2	4.8 ± 0.3	4.4 ± 0.2	4.3 ± 0.1	5.2 ± 0.4
Fasting plasma insulin (μU/l)	20.0 ± 1.1	23.2 ± 1.4	34.9 ± 3.7*	32.1 ± 2.2*	24.4 ± 2.0 [#]	19.2 ± 2.3 [#]

Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Data are expressed as mean ± standard error of mean of 6 rats per group (* $p < 0.05$ vs. control, [#] $p < 0.05$ vs. COC).

Table 2

Effects of Nicotine on hematological Parameters in COC-treated female rats.

	Control	Low Nicotine	High Nicotine	COC	COC+ Low Nicotine	COC+ High Nicotine
RBC (10^{12} cells/l)	7.1 ± 0.3	7.1 ± 0.3	7.5 ± 0.5	7.9 ± 0.3	6.9 ± 0.2 [#]	6.8 ± 0.3 [#]
Hb (g/l)	13.9 ± 0.3	14.3 ± 0.6	14.9 ± 0.6	15.7 ± 0.5*	13.9 ± 0.3 [#]	13.7 ± 0.6 [#]
PLT (10^9 cells/l)	379.7 ± 26.8	330.5 ± 50.7	373.8 ± 13.5	525.0 ± 28.6*	400.3 ± 40.5 [#]	461.0 ± 43.6
WBC (10^9 cells/l)	5.6 ± 0.3	10.6 ± 1.4*	6.0 ± 1.0	5.3 ± 1.5	6.3 ± 0.9	7.1 ± 1.9
PMN (10^9 cells/l)	1.0 ± 0.3	1.6 ± 0.1	0.9 ± 0.1	1.3 ± 0.3	1.0 ± 0.2	1.3 ± 0.2
LYMP (10^9 cells/l)	4.4 ± 0.3	8.3 ± 1.2*	4.9 ± 0.9	3.6 ± 1.1	3.9 ± 1.0	5.4 ± 1.5

Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Data are expressed as mean ± standard error of mean of 6 rats per group (* $p < 0.05$ vs. control, [#] $p < 0.05$ vs. COC).

RBC, red blood cell count; Hb, hemoglobin concentration; PLT, platelet count; WBC, white blood cell count; PMN, polymorphonuclear cell count; LYMP, lymphocyte count.









